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Wound-induced H_2O_2 and resistance to *Botrytis cinerea* decline with the ripening of apple fruit

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ABSTRACT

Fruit ripening is a developmental process and is associated with increased susceptibility to mechanical injury, which favours *Botrytis cinerea* infection. Using 'Gala' apples harvested at different stages of ripening, we demonstrated that wounding can activate initial H_2O_2 accumulation and wound healing ability to defend against *B. cinerea* penetration. Delaying the harvest date attenuated those responses. Superoxide dismutase, peroxidase and catalase, which are all involved in H_2O_2 metabolism, were differentially activated by wound stress depending on the stage of fruit maturity. Mature fruit were less able to respond to wounding by increasing phenylalanine ammonia lyase and peroxidase activity, which are associated with reduced phenolics and lignin content in local wound sites. The reduced response in late-harvested fruit contributes to the fruit ripening-induced loss of wound healing ability and increases susceptibility to *B. cinerea*. In addition, the rapid increase of H_2O_2 content immediately after wounding in early-harvested fruit was followed by increased phenylalanine ammonia lyase and peroxidase activity. In late-harvested fruit, the reduced ability to increase phenylalanine ammonia lyase and peroxidase activity in response to wounding was consistent with ripening-reduced generation of H_2O_2 early after wounding, leading to reduced resistance to *B. cinerea*. Thus, H_2O_2 accumulation in response to wounding is modulated by fruit maturity and is required for efficient wound healing and resistance to *B. cinerea*.

1. Introduction

Botrytis cinerea causes gray mold disease in a broad range of fruit types, including apples, and is one of the most severe postharvest diseases. In most cases, this pathogen requires a wound in the epidermis to enter susceptible tissue and initiate infection (Spotts et al., 1998). Mechanical injury, caused during harvesting and postharvest handling, provides an optimal locus for infection. The increased susceptibility to mechanical damage caused by fruit ripening, which leads to biochemical changes such as enhanced respiration and oxidative stress, cell wall breakdown and reduced protein synthesis, may lower resistance to pathogen penetration (Imaseki, 1985; Torres et al., 2003). Though plant tissue can induce multiple defense strategies to overcome wound stress and prevent further pathogen invasion, these wound-induced defense responses may be modulated by fruit ripening.

The early phase of a plant's response to wounding is usually accompanied by the generation of hydrogen peroxide, H_2O_2 (Orozco-Cańdenas and Ryan, 1999). The presence or accumulation of H_2O_2 at the wound surface has been postulated to play an important role in plant defense (Wu et al., 1995; Rea et al., 2002). In addition to its oxidative potential in killing or inhibiting the growth of pathogens, H_2O_2 has been implicated in reinforcing the cell wall around wounds by the oxidative cross-linking of apoplastic structural protein and lignin and suberin polymerization. H_2O_2 also serves as a second messenger in induction of defense genes (Wu et al., 1997). Despite its importance in wound healing, H_2O_2 accumulation by wounding may depend on development in the organism. In 'Golden Delicious' fruit, a two week harvest delay resulted in a lower increase in H_2O_2 levels after wounding, which was associated with higher susceptibility to *Penicillium expansum* (Torres et al., 2003). In potato tubers, age-induced loss in the ability of tubers to produce a burst of superoxide radicals is coincident with reduced capacity to develop a wound barrier effective against pathogen infection (Kumar and Knowles, 2003). Moreover, superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and/or peroxidase (POD, EC 1.11.1.7), which involved in H_2O_2 metabolism, are differentially activated by wound stress depending on plant growth stage (Chandru et al., 2003). Thus, we hypothesized that wound-induced H_2O_2 and associated enzymes that play an important role in wound-defense processes are also regulated by fruit ripening.

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The wound healing response results in the production of wound periderm, which was thought to be lacking in fruit after harvest (Simons and Aubertin, 1959; Skene, 1981). However, the accumulation of phenolics and lignin-like materials strengthen the cell wall around wounds in mature fruit and effectively protect the underlying tissue from invasion by pathogens (Lakshminarayana et al., 1987). Phenylalanine ammonia lyase (PAL; EC 4.3.1.5) is a key enzyme in the first step of the phenylpropanoid pathway, which is directly involved in the synthesis of phenols and lignin (Yao and Tian, 2005). Apples, a typically climacteric fruit, release autocatalytic ethylene as the fruit ripens. Since PAL can be induced by ethylene, more mature fruit with higher ethylene production may induce higher PAL activity and gain increasing resistance to pathogens. However, Torres et al. (2003) showed that late harvest mature fruit were more susceptible to *P. expansum*, which was associated with lower wound-induced H_2O_2. In potatoes, older tubers with increased accumulation of ethylene lack the ability to form superoxide radicals on wound surfaces and are less efficient at up-regulating PAL in response to wounding (Kumar and Knowles, 2003). These results raise questions about whether decreased resistance to pathogens in more mature apple fruit is a result of lower wound-induced H_2O_2 levels, leading to reduced wound healing ability, or rather the H_2O_2 serves as a signal to induce the expression of PAL. Thus, we examined the effects of fruit ripening in 'Gala' apples on ethylene production, PAL activity, and wound-induced H_2O_2, and analyzed their impact on wound healing ability and resistance to *B. cinerea*.

2. Materials and methods

2.1. Fruit

'Gala' apples (*Malus domestica* Borkh.) were obtained from a commercial orchard in Xuzhou (Jiangsu, China) and transferred within 24 h to our laboratory. Harvests were carried out on August 10 (H1, early), August 17 (H2, optimal) and August 24 (H3, late), 2010. Fruit were selected for uniform size and color without physical injuries or apparent infections. Harvest maturity indices were measured at each date.

2.2. Quality parameters

Firmness was measured on two opposite peeled sides using a penetrometer (FT 327, Facchini, Italy) with an 11 mm diameter probe. Soluble solids concentration (SSC) was determined by measuring the refractive index of the juice, and the data were expressed as a percentage, grams per 100 g fresh weight (FW). Acidity was measured as follows: 20 mL of pulp juice was diluted with 50 mL H_2O and titrated with 0.1 M NaOH solution to pH 8.10. The acidity was expressed in grams of malic acid per litre of juice. Starch Index was scored visually by using a 1–9 scale (1: high starch; 9: no starch), after staining an equatorial section with 0.22% (w/v) I_2–0.88% (w/v) KI solution. Data on maturity parameters represent the mean of 20 individual fruit. The same fruit were used to determine all the maturity indices.

2.3. Pathogen inoculum

B. cinerea was isolated from rotten apples during storage, and maintained on potato dextrose agar (PDA: infusion from potatoes 1000 mL, glucose 20 g, agar 15 g) at 4 °C. Fresh culture was grown on PDA plates at 25 °C before use. Spores were subsequently harvested by flooding the surfaces of 10-day old cultures with sterile distilled water and gently agitating the plates to dislodge the spores. The conidial suspension was prepared in 0.05% (v/v) Tween-80. Spore concentration was adjusted to 1×10^5 spores mL^{-1} with the aid of a haemocytometer.

2.4. Wounding, wound healing and pathogen inoculation

After harvest, 'Gala' apples were surface-sterilized with 2% sodium hypochlorite (v/v) for 2 min, rinsed with tap water and air-dried. All apples were wounded with a sterilized stainless-steel nail by making an injury 2 mm in diameter and 2–3 mm deep at two sides of each fruit, and incubated at 20 °C (85% RH) for 96 h. The healed wounds were then inoculated with 20 μL aqueous suspensions containing 10^5 conidia mL^{-1} *B. cinerea* and 0.05% (v/v) Tween-80 in each wound. Fresh wounds were inoculated immediately after wounding. The inoculated apples were stored at 20 °C, 85 ± 5% RH for ten days, after which the percentage of infected wounds and the lesion diameter caused by *B. cinerea* were measured. When the visible rot zone beyond the wound area on each fruit was more than 1 mm wide, it was counted as an infected wound. Ten apples (containing 20 wounds) were used for pathogen inoculation and the experiment was performed in triplicate.

2.5. Ethylene production after wounding

After each harvest, 'Gala' apples were wounded and incubated as described above. Ten fruit were enclosed in 10 L airtight jars for 2 h at 25 °C at the following time points: 0, 6, 12, 24, 48, 72 and 96 h after wounding. The headspace gas in the jars were sampled with a 1 mL plastic hypodermic syringe and injected into a gas chromatograph (model GC-14B, Shimazu, Japan) fitted with an alumina column at 70 °C and a flame ionization detector to assay the ethylene production. The rate of ethylene production was expressed as nL h^{-1} kg^{-1} FW. There were two replications for ethylene assays and the experiment was performed in triplicate.

2.6. Fruit tissue sampling

After harvest, 'Gala' apples were surface-sterilized and air-dried as described above. Four wounds were made at the equator of each fruit using the sterilized stainless-steel nail. Afterwards, all fruit were incubated at 20 °C (85% RH) for 96 h. Plugs of fruit tissue 1 cm diameter and 1 cm deep and centered around wounds were extracted using a cork borer from 10 fruit at 0, 6, 12, 24, 48, 72 and 96 h after wounding. Samples were mixed and frozen immediately in liquid nitrogen, then stored at −20 °C. Frozen samples were used for enzyme assays and measurements of protein, H_2O_2, total phenolic and lignin contents. There were two replications for each harvest date and the experiment was performed in triplicate.

2.7. Measurement of enzyme activity

All procedures of enzyme extraction were performed at 4 °C. For superoxide dismutase (SOD), 1 g of frozen tissue was ground with 5 mL 50 mM sodium phosphate buffer (pH 7.8) containing 0.1 mM ethylene diamine tetraacetic acid (EDTA) and 3% polyvinyl polypyrrolidone (PVPP) (w/v). For catalase (CAT), 1 g of frozen tissue was ground with 3 mL 100 mM sodium phosphate buffer (pH 7.0) containing 3% PVPP (w/v). For peroxidase (POD), 1 g of frozen tissue was ground with 3 mL 100 mM sodium phosphate buffer (pH 6.4) containing 3% PVPP (w/v). For phenylalanine ammonia lyase (PAL), frozen tissue (2 g) was ground with 5 mL of 100 mM sodium borate buffer (pH 8.7) containing 0.037% EDTA (w/v), 0.137% β-mercaptoethanol (v/v), and 3% PVPP (w/v). The extracts were then homogenized and centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatants were used for the enzyme assays.

SOD activity was determined by the method of Zhao et al. (2009) in a final volume of 3 mL, which contained 0.1 mL of

enzyme extract, 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM nitroblue tetrazolium (NBT), 10 μM EDTA, and 10 μM riboflavin, was illuminated with a fluorescent lamp (60 μmol m^{-2} s^{-1}) for 10 min, and the absorbance at 560 nm was recorded. An aliquot of identical solution was kept in the dark and served as the blank control. One unit of SOD activity is defined as the amount of enzyme that catalyzes 50% decrease of the SOD-inhibitable NBT reduction.

CAT activity was assayed according to the method of Zhao et al. (2009). The assay mixture contained 100 mM sodium acetate (pH 7.0), 25 mM H_2O_2 and 0.2 mL crude enzyme extract in a total volume of 3 mL. Decomposition of H_2O_2 was measured by reduction in absorbance at 240 nm (UV 1102 spectrophotometer, Shanghai, China). One unit of CAT was defined as the amount of enzyme that decomposes 1 μmol of H_2O_2 per min at 30 °C.

POD was assayed using the method of Cao et al. (2008) with some modifications. The assay mixture contained 100 mM sodium acetate (pH 6.4), 25 mM H_2O_2, 20 mM guaiacol and 0.2 mL crude enzyme extract in a total volume of 3 mL. POD activity was determined by measuring absorbance at 460 nm. One unit of POD activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 at 460 nm in 1 min.

PAL was assayed by the method described by Zhao et al. (2009) with some modifications. The assay medium contained 0.5 mL of enzyme extract, 1 mL of 10 mM L-phenylalanine and 1.5 mL of 100 mM sodium borate buffer (pH 8.7). After incubation at 37 °C for 1 h, the reaction was stopped by adding 0.2 mL of 6 N HCl. The activity was determined by measuring absorbance at 290 nm. One unit of PAL activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 at 290 nm in 1 h under the specified conditions.

Protein content in the enzyme extracts was estimated using the Bradford (1976) method, using bovine serum albumin as a standard. Specific activity of all the enzymes was expressed as units per milligram protein.

2.8. Measurements of H_2O_2, phenolics and lignin content in wounded tissue

For H_2O_2 contents, frozen tissue (2 g) was homogenized with 5 mL of chilled 100% acetone and then centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant was used immediately for H_2O_2 analysis by monitoring the absorbance of the titanium–peroxide complex at 410 nm according to the method of Patterson et al. (1984). Absorbance values were calibrated against a standard curve (generated using known concentrations of H_2O_2) and results were expressed as μmol g^{-1} FW.

For total phenolic contents, frozen tissue (2 g) was homogenized with 5 mL of chilled 80% ethanol and centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant was used for analysis. Total phenolic content was determined using the method of Cao et al. (2008). The results were expressed as milligrams of gallic acid equivalent (GAE) per 100 g FW.

Lignin was gravimetrically determined according to the methods of Assis et al. (2001) with some modification. Frozen tissue (10 g) was dispersed in 20 mL 72% H_2SO_4 at room temperature for 6 h then diluted to 1 M H_2SO_4 and heated for 2.5 h at 100 °C. Insoluble material was recovered by filtration and rinsed with 100 °C distilled water until acid free before drying at 105 °C for 4 h. The residue weight was recorded as lignin content and results were expressed as a percentage of FW.

2.9. Statistical analyses

Data were analyzed by one-way analysis of variance (ANOVA) to test the difference between harvest dates by using SAS software (Version 8.2; SAS Institute, Cary, NC, USA). Comparison of means was performed by Duncan's multiple range tests. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Changes in maturity indices

Quality parameters of 'Gala' apples changed significantly with harvest date (Table 1). Fruit firmness and acidity content decreased significantly ($P < 0.05$) with increasing maturity. Fruit from the latest harvest (H3) had the highest SSC value and starch index ($P < 0.05$).

Table 1
Effect of harvest date on maturity indices in 'Gala' apples.

	Firmness (N)	SSC (%)	Acidity (g L^{-1} malic acid)	Starch index
H1	101.0 a	11.4 c	3.24 a	4.1 c
H2	91.7 b	12.1 b	2.77 b	7.4 b
H3	74.5 c	13.4 a	2.44 c	8.6 a

Different letters in the same column are significantly different according to Duncan's multiple range test at $P = 0.05$ level.

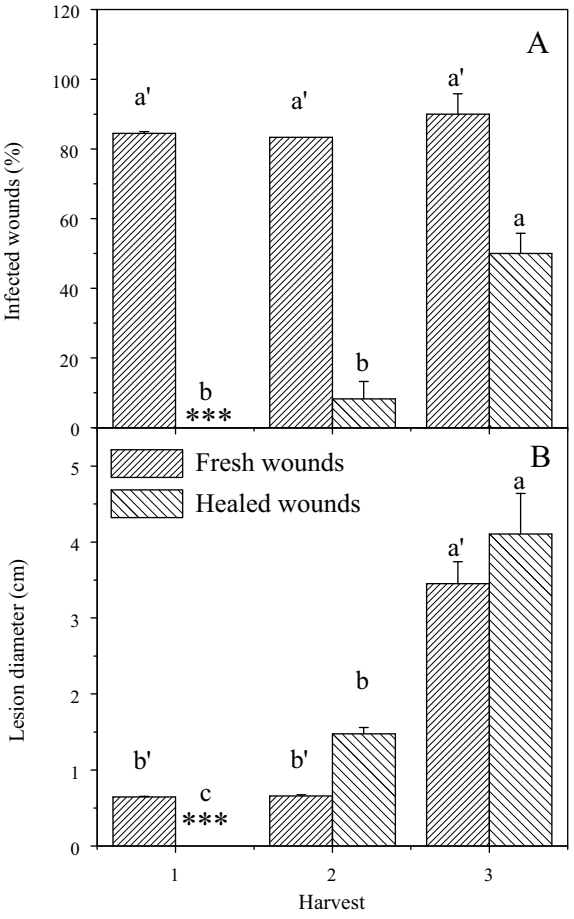

Fig. 1. Effects of fruit maturity on resistance to *B. cinerea* in wounds from harvest 1, 2 and 3 of 'Gala' apples following a 96 h period of wound-healing at 20 °C (85% RH). Fresh wounds and healed wounds were inoculated with 20 μL 10^5 conidia/mL aqueous suspensions of *B. cinerea*, and placed at 20 °C (85% RH), ten days for decay development. Each value represents the means of three replications with corresponding standard error. Columns with different letters were significantly different according to Duncan's multiple range test at $P = 0.05$ level. ***No decay development.

3.2. Effects on wound-healing

In order to assess wound healing ability, wounds of fruit from H1, H2 and H3 harvests were incubated at 20 °C for 96 h and inoculated with *B. cinerea*. In contrast to wounds from early (H1) and optimally (H2) harvested fruit, late (H3) harvested fruit showed a significantly ($P < 0.05$) higher gray mold decay development (Fig. 1). The gray mold decay incidence from fresh wounds was more than 80% and showed minimal increase with fruit ripening. However, healed wounds showed a 5-fold increase in decay incidence with delaying fruit harvest (Fig. 1A). Healed wounds from H3 fruit lost resistance to *B. cinerea* faster than those from H1 and H2 fruit, reflecting a significant reduction in wound healing ability of more mature fruit. The lesion diameter of *B. cinerea* in both healed and fresh wounds showed a similar pattern of increase with fruit ripening, but the increased rate in healed wounds was significantly higher ($P < 0.05$) than that in fresh wounds (Fig. 1B).

3.3. Effects on wound-induced H_2O_2 and associated enzymes activity

Significant differences in the kinetics of H_2O_2 accumulation in wound tissue were found for fruit harvested at different dates (Fig. 2A). Early harvested fruit (H1) exhibited a 1.6 fold increase in H_2O_2 content during the first 12 h after wounding, higher than that in optimally harvested fruit (H2). Later, these levels both decreased in H1 and H2 fruit. In contrast, late harvested fruit (H3) showed a gradual increase in H_2O_2 level during healing time.

The initial SOD activity (time 0) increased significantly ($P < 0.05$) as fruit maturity increased from H1 to H3. SOD activity increased rapidly for the first 6 h after wounding and maintained a higher level throughout the healing period for all fruit (Fig. 2B). Harvest date of apple fruit showed no effect on the increase pattern of wound-induced SOD activity. The rate of increase however, was significantly ($P < 0.05$) higher in wound tissue from early harvested fruit (H1) as compared with that from late harvest fruit (H3).

In early harvested fruit (H1), wound stress led to a decrease in CAT activity in the first 48 h and then showed a significant ($P < 0.05$) increasing pattern (Fig. 2C). Thus, transient deactivation of CAT resulted in H_2O_2 accumulation during the first phase of wound response. In contrast, POD activity increased until 12 h and then decreased, but increased rapidly between 48 and 96 h during the healing period (Fig. 3D). In the optimally harvested fruit (H2), CAT and POD activity decreased transiently immediately after wounding and then increased quickly. In late harvested fruit (H3), CAT and POD activity both decreased quickly after wounding. This decline in the activity of CAT and POD in late harvested fruit was coincident with an increase accumulation of H_2O_2 in wound tissue (Fig. 2).

3.4. Effects on ethylene production and PAL activity in wounding

The internal ethylene concentration of fruit showed a 2.1 fold increase as fruit maturity increased from H1 to H3 (Fig. 3A). Ethylene production increased linearly ($R = 0.983$) with time after wounding in late harvested fruit (H3). In optimally harvested fruit (H2), the increase in rate of ethylene production was lower when compared with that in late harvested fruit (H3). However, significant increases in ethylene production were not evident in early harvested fruit (H1). Regardless of the stage of maturity, PAL activity increased rapidly immediately after wounding (Fig. 3B). The rate of increase was significantly higher in wounded tissue from early harvested immature fruit (H1) as compared with that from optimally (H2) and late harvested (H3) fruit. This fruit ripening-reduced ability to increase PAL activity in response to wounding was coincident with the rapid increase in ethylene production (Fig. 3).

3.5. Changes in phenolics and lignin contents

Harvest date affected the accumulation of phenolic production in wound tissue of apple fruit (Fig. 4A). Phenolics were quickly increased within 6 h after wounding in early (H1) and optimally harvested (H1) fruit. Later, these levels both decreased. In compar-

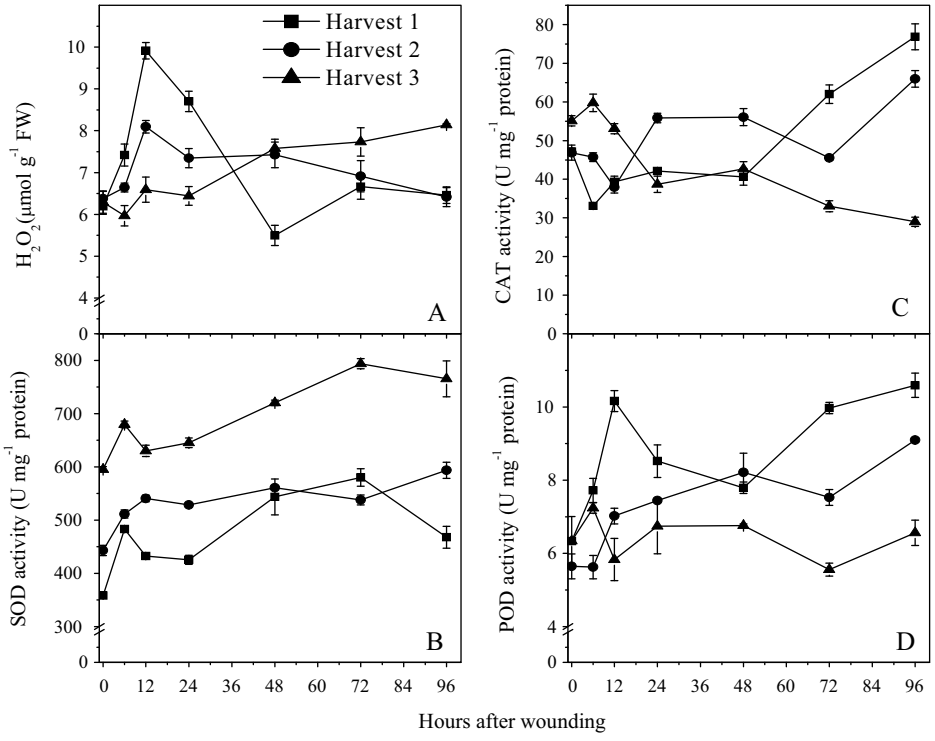

Fig. 2. Effects of wounding on H_2O_2 production (A), SOD (B), CAT (C) and POD (D) activity in 'Gala' apple fruit from different harvests. Data are expressed as mean ± S.E ($n = 6$).

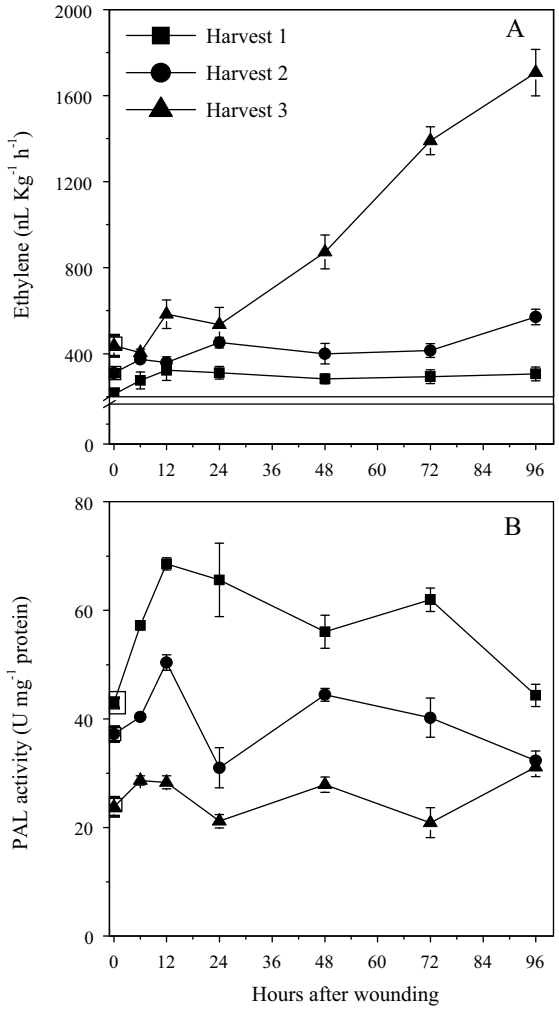

Fig. 3. Changes in ethylene production and PAL activity during the wound healing period in 'Gala' apple fruit wounded at different harvest dates. Data are expressed as mean ± S.E (n = 6).

ison with optimally harvested fruit (H2), early harvested immature fruit (H1) showed a higher level of phenolic production during the healing period. The internal phenolic production in late harvested fruit (H3) was significantly lower than those in immature and overly mature fruit (Fig. 4A), but increased steadily after wounding. As the delay in harvest date, wounded fruit showed a lower increase in lignin accumulation (Fig. 4B). The decrease of phenolic production in wound tissue from immature and overly mature fruit coincided with an increased formation of lignin in the second phase of wound healing (Fig. 4). In contrast, the increased accumulation of phenolic production in wound tissue from late harvested fruit (H3) was associated with a decreased accumulation of lignin.

4. Discussion

The maturity of a fruit alters its wound healing ability, and apple fruit ripening is a typical example of the dramatic developmental transition that has allowed analysis of changes in plant responses to wound stress. We found that successive one-week delays in harvest of 'Gala' apples resulted in significant differences in wound resistance to *B. cinerea* (Fig. 1). Gray mold decay incidence in fresh wounds was similar in fruit harvested at different dates, but the severity of decay increased significantly in more mature fruit after a wound healing period. Thus, maturity increased fruit susceptibility to *B. cinerea* from wounds due to the reduced wound-induced defense resistance. The decrease in firmness and increase in sugar concentrations observed in the mature fruit may play a minor role in the incidence of *B. cinerea* infection. However, these factors mainly favor the growth of *B. cinerea* lesions following infection, because the lesion diameter increased rapidly with fruit ripening in both fresh and healed wounds (Fig. 1B). In addition, fruit senescence during the healing period may increase wound susceptibility to *B. cinerea*, leading to increased lesion diameter.

The early generation of H_2O_2 in plant response to wounding has been postulated to play an important role in plant defense (Rea et al., 2002; Patykowski, 2006). The timely generation of H_2O_2 in response to wounding was necessary for induced resistance in winter squash (Watanabe et al., 2001) and tomato leaves (Orozco-Cańdenas and Ryan, 1999). In apple fruit, the lower susceptibility of early harvested fruit to *P. expansum* infection was correlated with the induction of H_2O_2 production (Torres et al., 2003). Macarisin et al. (2007) also found that the suppression of H_2O_2 production by citric acid and exogenous CAT disrupt host defenses, making citrus fruit susceptible to the non-host pathogen *P. expansum*. The current study showed that H_2O_2 generation occurs in early and optimally harvested apple fruit immediately after wounding and it continues for up to 12 h and decreases thereafter. Further, harvest maturity alters this timely generation of H_2O_2 upon wounding. The reduced resistance in healed wounds caused by fruit ripening (Fig. 1A) is coincident with a decline in the ability to produce H_2O_2 immediately after wounding (Fig. 2A). This supports the previous study that demonstrated late-harvest fruit with less increased levels of

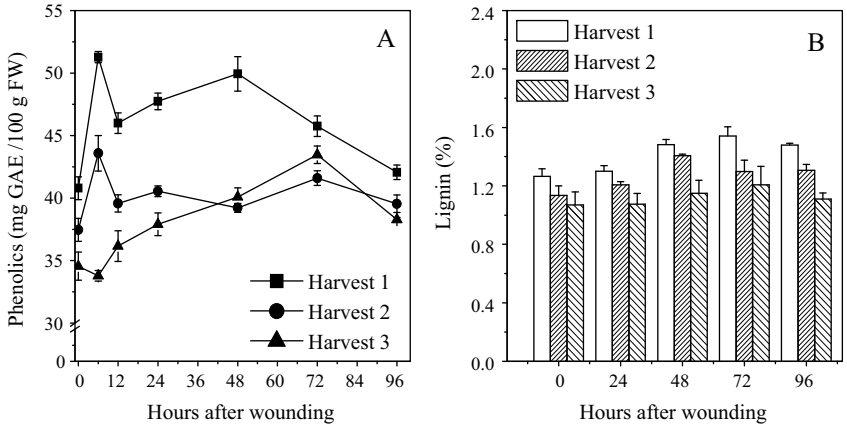

Fig. 4. Changes in phenolics and lignin content during the wound healing period in 'Gala' apple fruit wounded at different harvest dates. Data are expressed as mean ± S.E (n = 6).

H_2O_2 upon wounding were associated with disease susceptibility (Torres et al., 2003). Moreover, the positive increase of H_2O_2 content from 24 h to 96 h after wounding was observed in late harvested fruit, this may be lethal to host cells adjacent to wounds, leading to decreased wound resistance.

Given its limited life and toxicity potential, H_2O_2 must be generated in situ and its level must be finely regulated. Our results showed that the antioxidant enzymes involved in H_2O_2 metabolism were differentially activated by wound stress depending on the stage of fruit maturity. $O_2^{\bullet -}$ dismutation by SOD may be the important source of H_2O_2 in plants. Previous findings suggest that SOD participates in the defense mechanism of apple and citrus fruit against pathogen infection (Torres et al., 2003; Ballester et al., 2006). We demonstrated that SOD was activated in all maturity stages of apple fruit (Fig. 2B) and this may contribute to decreased susceptibility of healed wounds to *B. cinerea*. The key enzymes involved in H_2O_2 scavenging are CAT and/or POD. In this present research, high accumulation of H_2O_2 immediately after wounding in early and optimal harvested fruit was accompanied by elevated total SOD activity and decreased CAT activity in wound tissue in the first phase of wound healing (Fig. 2A–C). Subsequently, the rapid increase in CAT and POD activity during the later phase of wound healing may promote the elimination of H_2O_2 in wound tissue of early and optimal harvested fruit, which protect cells around wounds from oxidative damage. Though, the initial activity of SOD increased substantially (1.7-fold) from early to late harvested fruit, the notably higher activity of CAT at the first 12 h in wound tissue from late harvested fruit may limit the accumulation of H_2O_2. POD forms a complex family of proteins that catalyze the oxidoreduction of various substrates like phenolics using H_2O_2. Recent findings have indicated that extracellular POD may also generate H_2O_2 as a consequence of its reactions (Bolwell et al., 2002; Mika et al., 2004). We observed that increase in POD activity immediately after wounding correlated with high H_2O_2 levels in wound tissue of early harvested fruit. This finding is in agreement with previous suggestion that the activation of POD in harvested fruit in response to postharvest biocontrol agents may also have been associated with an increase in host levels of H_2O_2 and concomitant host defense response (Macarisin et al., 2010).

The wound healing process is slower than the oxidative burst in plant response to wounding. Fruit after harvest exhibit a positive wound healing response by local accumulation of phenolics and lignin in cell wall thickening around wounds (Lakshminarayana et al., 1987; Spotts et al., 1998). Our results provide strong evidence that a 96 h healing period enhanced accumulation of phenolics and lignin in wounds of apple fruit, which increased resistance to *B. cinerea* when compared with fresh wounds. The rate of increase however, was significantly higher in wounded tissue from early harvested fruit as compared with that from late harvest fruit (Fig. 4). PAL, a key enzyme in the first step of the phenylpropanoid pathway, is directly involved in the synthesis of phenols and lignin that are correlated with localized resistance processes. Ethylene can induce the de novo synthesis of PAL (Kato et al., 2000), which in turn synthesizes *t*-cinnamic acid that was oxidatively cross-linked by POD/H_2O_2 into lignin. Late harvested fruit contained higher concentrations of ethylene than early harvested fruit (Fig. 3A). After wounding, late harvested fruit also showed a significantly higher increase in rate of ethylene production when compared with those in early and optimal harvested fruit. This rapid increase in ethylene production as fruit ripen corresponds with the reduced ability to increase PAL activity in response to wounding (Fig. 3). This finding correlates with the reports from potato tuber, that advancing tuber age with higher ethylene concentration lead to a loss in the ability to produce PAL activity in response to wounding (Kumar and Knowles, 2003). Fruit ripening can be considered a form of regulated senescence, and ethylene promotes ripening and senescence in fruit (Cantu et al., 2009). Thus, the reduced ability of increasing PAL activity in response to wounding may be due to a reduced synthesis of PAL caused by fruit senescence. Recently, the role of H_2O_2 as a signaling molecule in inducing plant defense genes has been well established (Wu et al., 1997; Desikan et al., 2000). In *Arabidopsis* suspension cultures, H_2O_2 can induce the expression of GST and PAL (Desikan et al., 1998). Suppressing the production of H_2O_2 by dimethylthiourea can decrease the PAL activity in rice cells (Lin et al., 2005). In our study, the reduced ability to increase PAL in response to wounding caused by fruit ripen (Fig. 3B) was consistent with the reduced generation of H_2O_2 in the early phase of wounding (Fig. 2A), indicating that H_2O_2 may induce PAL expression. In addition, our results demonstrated that phenolic productions accumulated within 24 h after wounding, consistent with the increase of PAL activity in early and optimally harvested fruit's response to wounding. POD plays an important role in the polymerization of phenolics. The increase in POD activity and decrease in soluble phenolics from 48 to 96 h after wounding in early and optimally harvested fruit may represent their polymerization by POD, and correspondingly, lignin content in early and optimally harvested fruit was significantly higher than in late harvested fruit 48 to 96 h into the wound healing period. Late-harvested fruit exhibited lower phenolics and lignin content in local wound tissue. This was associated with lower PAL and POD activity in response to wounding, when compared with early and optimally harvested fruit. Therefore, the reduced capacity for phenolics and lignin accumulation at wound sites in late harvested fruit may contribute to the ripening induced loss in wound healing ability and resistance to *B. cinerea*.

In conclusion, we demonstrated that: (1) the ability to produce H_2O_2 immediately after wounding and the efficiency of healing declined with fruit ripening, which resulted in increased wound susceptibility to *B. cinerea*; (2) SOD is activated at all stages of fruit maturity, and CAT and POD are differentially activated depending on fruit maturity, in response to wounding; (3) the reduced wound healing ability resulting from fruit ripening is positively correlated with the reduced ability to produce PAL and POD in response to wounding, and limited phenolics and lignin accumulation in local wounds. Moreover, an increase in H_2O_2 production in response to wounding is followed by the induction of PAL. Thus, H_2O_2 accumulation in response to wounding is modulated by fruit maturity and required for efficient wound healing.

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References

Assis, J.S., Maldonado, R., Muñoz, T., Escribano, M.I., Merodio, C., 2001. Effect of high carbon dioxide concentration on PAL activity and phenolic contents in ripening cherimoya fruit. Postharvest Biol. Technol. 23, 33–39.

Ballester, A.R., Lafuente, M.T., González-Candelas, L., 2006. Spatial study of antioxidant enzymes, peroxidase and phenylalanine ammonia-lyase in the citrus fruit-*Penicillium digitatum* interaction. Postharvest Biol. Technol. 39, 115–124.

Bolwell, G.P., Bindschedler, L.V., Blee, K.A., Butt, V.S., Davies, D.R., Gardner, S.L., Gerrish, C., Minibayeva, F., 2002. The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. J. Exp. Bot. 53, 1367–1376.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle-dye binding. Anal. Biochem. 72, 248–254.

Cantu, D., Blanco-Ulate, B., Yang, L., Labavitch, J.M., Bennett, A.B., Powell, A.L.T., 2009. Ripening-regulated susceptibility of tomato fruit to *Botrytis cinerea* requires *NOR* but not *RIN* or ethylene. Plant Physiol. 150, 1434–1449.

Cao, S.F., Zheng, Y.H., Yang, Z.F., Tang, S.S., Jin, P., Wang, K.T., Wang, X.M., 2008. Effect of methyl jasmonate on the inhibition of *Colletotrichum acutatum* infection in loquat fruit and the possible mechanisms. Postharvest Biol. Technol. 49, 301–307.

Chandru, H.K., Kim, E., Kuk, Y., Cho, K., Han, O., 2003. Kinetics of wound-induced activation of antioxidative enzymes in *Oryza sativa*: differential activation at different growth stages. Plant Sci. 164, 935–941.

Desikan, R., Reynolds, A., Hancock, J.T., Neill, S.J., 1998. Harpin and hydrogen peroxide both initiate programmed cell death but have differential effects on gene expression in *Arabidopsis* suspension cultures. Biochem. J. 330, 115–120.

Desikan, R., Neill, S.J., Hancock, J.T., 2000. Hydrogen peroxide-induced gene expression in *Arabidopsis thaliana*. Free Radic. Biol. Med. 28, 773–778.

Imaseki, I., 1985. Hormonal Regulation of Plant Development (III): Control of Wound-Induced Responses. SpringerVerlag, Berlin, Germany.

Kato, M., Hayakawa, Y., Hyodo, H., et al., 2000. Wound-induced ethylene synthesis and expression and formation of 1-aminocyclopropane-1-carboxylate (ACC) synthase, ACC oxidase, phenylalanine ammonia-lyase, and peroxidase in wounded mesocarp tissue of *Cucurbita maxima* [J]. Plant Cell Physiol. 41 (4), 440–447.

Kumar, M.G.N., Knowles, R.N., 2003. Wound-induced superoxide production and PAL activity decline with potato tuber age and wound healing ability. Physiol. Plant 117, 108–117.

Lakshminarayana, S., Sommer, N.F., Pollito, V., Fortlage, R.J., 1987. Development of resistance to infection by *Botritis cinerea* and *Pencillium expansum* in wounds of mature apple fruits. Phytopathology 77, 1274–1678.

Lin, W., Hu, X., Zhang, W., Rogers, W.J., Cai, W., 2005. Hydrogen peroxide mediates defense responses induced by chitosans of different molecular weights in rice. J. Plant Physiol. 162, 937–944.

Macarisin, D., Cohen, L., Eick, A., Rafael, G., Belausov, E., Wisniewski, M., Droby, S., 2007. *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit. Phytopathology 97, 1491–1500.

Macarisin, D., Droby, S., Bauchan, G., Wisniewski, M., 2010. Superoxide anion and hydrogen peroxide in the yeast antagonist–fruit interaction: a new role for reactive oxygen species in postharvest biocontrol? Postharvest Biol. Technol. 58, 194–202.

Mika, A., Minibayeva, F., Beckett, R., Lüthje, S., 2004. Possible functions of extracellular peroxidases in stress-induced generation and detoxification of active oxygen species. Phytochem. Rev. 3, 173–193.

Orozco-Cárdenas, M.L., Ryan, C.A., 1999. Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. Proc. Natl. Acad. Sci. U.S.A. 96, 6553–6557.

Patterson, B.D., MacRae, E.A., Ferguson, I.B., 1984. Estimation of hydrogen peroxide in plant extracts using titanium. Anal. Biochem. 139, 487–492.

Patykowski, J., 2006. Role of hydrogen peroxide and apoplastic peroxidase in tomato–*Botrytis cinerea* interaction. Acta Physiol. Plantarum 28, 589–598.

Rea, G., Metoui, O., Infantino, A., Federico, R., Angelini, R., 2002. Copper amine oxidase expression in defense responses to wounding and *Ascochyta rabiei* invasion. Plant Physiol. 128, 865–875.

Simons, R.K., Aubertin, M.P., 1959. Development of epidermal, hypodermal, and cortical tissues in the 'Golden Delicious' apple as influenced by induced mechanical injury. Proc. Am. Soc. Hortic. Sci. 74, 1–9.

Skene, D.S., 1981. Wound healing in apple fruits: the anatomical response of 'Cox's Orange Pippin' at different stages of development. J. Hortic. Sci. 56, 145–153.

Spotts, R.A., Sanderson, P.G., Lennox, C.L., Sugar, D., Cervantes, L.A., 1998. Wounding, wound healing and staining of mature pear fruit. Postharvest Biol. Technol. 13, 27–36.

Torres, R., Valentines, M.C., Usall, J., Viñas, I., Larrigaudiere, C., 2003. Possible involvement of hydrogen peroxide in the development of resistance mechanisms in 'Golden Delicious' apple fruit. Postharvest Biol. Technol. 27, 235–242.

Watanabe, T., Seo, S., Sakai, S., 2001. Wound-induced expression of a gene for 1-amino- cyclopropane-1-carboxylate synthase and ethylene production are regulated by both reactive oxygen species and jasmonic acid in *Cucurbita maxima*. Plant Physiol. Biochem. 39, 121–127.

Wu, G., Shortt, B.J., Lawrence, E.B., Levine, E.B., Fitzsimmons, K.C., Shah, D.M., 1995. Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell 7, 1357–1368.

Wu, G., Shortt, B., Lawrence, J., Fitzsimmons, K., Levine, E., Raskin, I., Shah, D., 1997. Activation of host defense mechanisms by elevated production of H_2O_2 in transgenic plants. Plant Physiol. 115, 427–435.

Yao, H.J., Tian, S.P., 2005. Effects of a biocontrol agent and methyl jasmonate on postharvest diseases of peach fruit and the possible mechanisms involved. J. Appl. Microbiol. 98, 941–995.

Zhao, Y., Tu, K., Su, J., Tu, S., Hou, Y., Liu, F., Zou, X., 2009. Heat treatment in combination with antagonistic yeast reduces diseases and elicits the active defense responses in harvested cherry tomato fruit. J. Agric. Food Chem. 57, 7565–7570.